Cranberry extract supplementation exerts preventive effects through alleviating Aβ toxicity in Caenorhabditis elegans model of Alzheimer’s disease

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Available online 20 June, 2016

[ABSTRACT] Cranberry extract (CBE) rich in polyphenols are potent to delay paralysis induced by alleviating β-amyloid (Aβ) toxicity in C. elegans model of Alzheimer’s disease (AD). In order to better apply CBE as an anti-AD agent efficiently, we sought to determine whether preventive or therapeutic effect contributes more prominently toward CBE’s anti-AD activity. As the level of Aβ toxicity and memory health are two major pathological parameters in AD, in the present study, we compared the effects of CBE on Aβ toxicity and memory health in the C. elegans AD model treated with preventive and therapeutic protocols. Our results revealed that CBE prominently showed the preventive efficacy, providing a basis for further investigation of these effects in mammals.

[KEY WORDS] Aβ toxicity; Cranberry polyphenols; Prevention; Body paralysis; Caenorhabditis elegans


Introduction

Aging is an inevitable process occurring in most of multicellular organisms [1-4]. Along with aging, the accumulation of damages at the molecular, cellular, and organ levels causes numerous declines in physiological, immunological, and cognitive functions, which in turn, may result in age-related disorders, such as Alzheimer’s disease (AD). AD is the most common neurodegenerative disease characterized by progressive loss of memory and impairment of cognitive ability in the elderly. One of the pathological hallmarks of AD is the accumulation of β-amyloid (Aβ) or Aβ-like proteins in the brain. Considering that AD has become a major public health concern worldwide, it is critical to find novel approaches to halt or delay the onset of AD [5-8]. Many lines of evidence have suggested that diet has an enormous impact on age-related disorders in almost all organisms including humans [9-11]. Nutraceuticals derived from natural dietary sources, such as those from blueberry, curcumin, and Gingko, may possess immense potential to combat age-related disorders, including AD [12-15].

Although nutraceutical consumption is a non-genetic way to treat age-related disorders, bioactive components in nutraceuticals may be functioning through genetic regulators, such as insulin signaling pathway, daf-16/FOXOs, and heat shock factors [1-2, 4]. Given that AD is associated with aging, the development of AD may be regulated by genes modulating lifespan and healthspan, which are expressed in a temporally and spatially regulated manner. Thus, the same functional nutraceutical may confer diverse anti-AD efficacy if supplemented at different stages in animals’ life. In addition, as Aβ toxicity, one of the major proteotoxities in AD, may significantly interfere with the function of some lifespan/healthspan-related genes, it is conceivable that the anti-AD efficacy of a particular nutraceutical may have dramatic difference when supplemented before or after the expression of Aβ peptide in AD animals. Therefore, it is interesting to determine whether nutraceutical supplementation alleviates Aβ toxicity relies more on the preventive (before Aβ
expression) or therapeutic (after Aβ expression) efficacies.

The North American cranberry (Vaccinium macrocarpon) and its products have been used extensively as nutraceuticals owing largely to their anti-microbial, anti-mutagenic, anti-angiogenic, and anti-oxidant properties [16-17]. We have previously reported that consumption of a cranberry extract (CBE) rich in polyphenols significantly promote healthy lifespan in Caenorhabditis elegans and Drosophila melanogaster [18-19]. In the present study, we investigated if CBE supplementation could attenuate Aβ toxicity in CL4176, a C. elegans model of AD. CL4176 is a transgenic worm line, in which human Aβ peptides can be expressed by heat-shock induction at the L3 larval stage [20-21]. Although studies have demonstrated that long-term supplementation of CBE benefits the AD treatment, whether application of CBE in prevention treatment and therapeutic treatment may show diverse efficacy remains unknown. In the present study, the preventive treatment was defined as that CBE supplementation started from egg hatching to L3 stage before Aβ expression in CL4176 worms was induced, while therapeutic treatment was defined as that CBE supplemented started at L3 stage when Aβ expression was induced. Accordingly, we examined the anti-AD effects of CBE in CL4176 AD models by altering the way of CBE treatment. Our data suggested that preventive treatment prominently delayed the body paralysis in CL4176 AD worms. Given that the body paralysis is triggered by Aβ toxicity, we measured the expression level of Aβ polypeptide in AD worms when treated with preventive and therapeutic protocols, respectively. Our results indicated that the preventive treatment led to more reduction of Aβ expression in AD worms as compared to the therapeutic treatment, suggesting that CBE may play a more critical role in prevention to combat against AD. Moreover, we further examined whether different CBE treatment protocols, preventive and therapeutic, may also confer different effectiveness to AD worms' memory. We found that CBE preventive treatment significantly improved the memory of AD worms compared with the therapeutic treatment. Taken together, our findings revealed that relative to the therapeutic efficacy, CBE-induced anti-AD effects may prominently ascribe to its efficacy of prevention in C. elegans.

As a powerful genetic model system, C. elegans AD model has been used to investigate gene expression and characterize gene function in response to Aβ toxicity [21-23]. To our best knowledge, we were the first to utilize C. elegans to assess CBE’s efficacy of prevention and cure on AD treatment. Outcomes from this research may have immediate implications to use CBE as a preventive agent to treat AD patients in the clinic. Meanwhile, considering that human system is much more complex than C. elegans, further studies in mammalian system need to be performed.

Materials and Methods

CBE preparation

The CBE rich in polyphenols, kindly provided by Naturex-DBS, LLC (Sagamore, MA, USA), was described in a previously published study [18, 24]. To prepare cranberry supplemented food, the CBE powder was dissolved in sterile distilled water. A fresh 10% (W/V) stock solution was prepared one day before the assay and the appropriate dilutions were then overlaid onto nematode growth medium (NGM) plates.

C. elegans maintenance

Wild type N2 worms and CL4176 [dvhs27[pA4F29(myo-3:: Aβ1-42-let 3′UTR) + [pRF4(rol-6(su1006))] (AD worm model)] were obtained from the Caenorhabditis Genetics Center, University of Minnesota, Minneapolis, MN, USA and maintained at 16 °C on NGM seeded with E. coli OP50 feeding strain. 100 μL of E. coli OP50 was dropped on the center of 60-mm NGM plates, which were allowed to dry overnight before worms were transferred.

Worm paralysis assay

As worm body paralysis is triggered by Aβ toxicity, a paralysis assay was used to indicate the alterations of Aβ toxicity. The assay using strain CL4176 was carried out as described by Dostal et al [20] with modifications. For CBE preventive treatment, staged populations of CL4176 transgenic worms were prepared by laying synchronous egg on NGM plates containing CBE. When eggs hatched and developed into L3 stage, they were transferred to regular NGM plates without CBE and induced to express Aβ via an upshift from 16 to 25 °C. For CBE therapeutic treatment, staged populations of CL4176 transgenic worms were prepared by laying synchronous egg on regular NGM plates. When eggs hatched and developed into L3 stage, they were transferred to NGM plates containing CBE and induced to express Aβ via an upshift from 16 to 25 °C. The CL4176 worms on regular NGM plates without CBE exposure served as negative controls. In contrast, the CL4176 worms on NGM plates with CBE exposure from egg until paralysis served as positive controls. All paralysis plots were done in triplicate with 15–20 worms per condition. Nematodes were scored as paralyzed if they exhibited ‘halos’ of cleared bacteria around their heads (indicative of insufficient body movement to access food) or failed to undergo a full body wave propagation upon prodding. The worms were checked every 2 h (start at 24 h after temperature upshift) and the worms that failed to move forward upon the tail prodding scored as paralyzed. The data were pooled and the percentage of non-paralyzed worms was calculated and analyzed using Student’s t-test. P < 0.05 was accepted as statistically significant.

Gene expression analysis by quantitative real-time PCR (qRT-PCR)

The CL4176 worms were synchronized by allowing 10–15 hermaphrodites lay eggs overnight on CBE containing NGM plates (for preventive treatment) or regular NGM plates (for therapeutic treatment) at 16 °C. The CL4176 worms on regular NGM plates without CBE exposure served as control, while the CL4176 worms exposed to CBE on NGM plates from egg until paralysis served as positive controls. The
worms at L3 stage were transferred to regular NGM plates (for preventive treatment) or CBE containing NGM plates (for therapeutic treatment), respectively, then upshifted from 16 to 25 °C. After a 24-h incubation, the worms were collected with M9 buffer into a 50–100 µL pellet. Total RNA was prepared using RNeasy RT reagent (Molecular Research Center, INC, Cincinnati, OH, USA) and stored at −80 °C. Complementary DNA was prepared by using Invitrogen Superscript first strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using SensiFAST SYBR No-Rox Kit (Bioline, Taunton, MA, USA) and the CFX96 real-time PCR detection system according to the manufacturer’s suggested protocol (Bio-Rad, Hercules, CA, USA). The qRT-PCR conditions were as follows: 95 °C for 3 minutes, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. Act-1 was used as an internal control and the Aβ1-42 RNA level was normalized to the level of act-1 for comparison. The qRT-PCR experiment was repeated three times using independent RNA/cDNA preparations. The data were pooled and analyzed using student’s t-test, and P < 0.05 was accepted as statistically significant. The qRT-PCR primers for Aβ1-42 were: 5′-CTTCT-GGCCACCAGCAGGTAC-3′ (Forward) and 5′-CTTGACAACCTTCGCCTGCTAG-3′ (Reverse). Primers for act-1 were: 5′-CCAGGAATTTGCTGATGTCAGAAGA-3′ (Forward) and 5′-TGGAGAGGGAAGCGAGGATAGA-3′ (Reverse).

Western blot analysis

The synchronous CL4176 worms treated with CBE were upshifted from 16 to 25 °C. The CL4176 worms on regular NGM plates without CBE exposure served as control, while the CL4176 worms exposed to CBE on NGM plates from egg until paralysis served as positive controls. After a 24-h incubation, the worms were transferred to microcentrifuge tubes and washed with S-basal. The total proteins were fractionated by standard SDS-PAGE and the proteins were transferred onto PVDF (BioRad) membranes. The membranes were incubated with primary antibodies (Cell Signaling Technology, #2454; diluted 1:1,000) in 5% non-fat dry milk and then with secondary, HRP-conjugated goat anti-rabbit antibodies (Genscript, Piscataway, NJ, USA, A00098; diluted 1:10,000). The protein detection was undertaken with standard ECL protocol. B-actin was used as loading control. The anti-ß antibodies (MAB1501) were from EMD Millipore Billerica, MA, USA. Mean intensity of Aß signals was analyzed using Image-J software (National Institute of Health, Bethesda, MD, USA).

Memory assays

As memory health is one major pathological parameter of AD, we performed the memory assay to assess CBE’s anti-AD effect. The CL4176 worms were maintained on OP50 seeded NGM plates for three generations at 16 °C, and then synchronized by egg laying on CBE containing NGM plates (for preventive treatment) and regular NGM plates (for therapeutic treatment), respectively. The worms at L3 stage were collected, washed with M9 buffer thrice, and then transferred to the worm area on regular NGM plates (for preventive treatment) or CBE containing NGM plates (for therapeutic treatment). Following temperature shift from 16 to 25 °C (to induce Aß expression), the worms’ migration toward food area was observed until all worms were paralyzed. The CL4176 worms without CBE exposure served as negative controls, while the CL4176 worms supplemented with CBE from egg to paralysis were setup as positive controls. For CBE preventive treatment, the assay plates were regular NGM plates without CBE, while the NGM plates containing CBE were used as assay plate for therapeutic treatment. Under each treatment, the number ratio of worms in OP50 food area verse total worms was used to assess the health of worms’ memory. Each assay was carried out in three independent trials. The data were pooled and analyzed using Student’s t-test. P < 0.05 was accepted as statistically significant.

Statistical analysis

Comparisons between two groups were performed using a two-tailed Student’s t-test assuming equal variances. Data from worm paralysis assays, qRT-PCR, and memory assays, were pooled to analyze, respectively Values of P < 0.05 was accepted as statistically significant.

Results and Discussion

Preventive treatment with CBE shows higher potency in delaying the progression of body paralysis in AD worms than therapeutic treatment

To determine the more effective way of CBE supplementation in AD treatment, we compared the efficacy of preventive treatment and therapeutic treatment to delay the body paralysis in the CL4176 AD worms. Since it has been reported that 2 mg·mL⁻¹ of CBE is able to significantly promote C. elegans healthspan [18, 25-26], we used the same concentration in the present study to carry out paralysis assays. The treatment methods are summarized in Fig. 1. The preventive treatment initialized the CBE treatment prior to eggs hatching and continued to L3 stage until heat-shock induction. The therapeutic treatment began the CBE treatment from L3 stage after heat-shock induction and continued until worms were paralyzed. The CL4176 worms were allowed to express Aß peptides for 24 h after heat shock, and then were checked for paralyses every 2 h until all worms were paralyzed. The CL4176 worms treated with CBE from eggs until body paralysis (long-term treatment) and without CBE served as positive and negative controls, respectively. All CBE treatments (prevention, therapy, and long-term) could delay the body paralysis of AD worms as compared to negative controls (Fig. 2). Notably, relative to CBE therapeutic treatment, the preventive treatment resulted in a dramatic delay in worm paralysis, which was more similar to CBE long-term treatment (Fig. 2), suggesting that the anti-AD
The effect of CBE was largely dependent on prevention. Considering that long-term treatment showed the best effect to delay AD worm paralysis, along with that the therapeutic treatment also contributed to delaying paralysis, our results further indicated that, in order to fully exert CBE’s anti-AD effects, CBE supplementation should not only start early before Aβ toxicity was generated, but also last in a long-term treatment.

**Fig. 1** Schematic presentation of the long-term treatment, preventive treatment and therapeutic treatment of CBE in the present study

**Fig. 2** CBE supplementation delays Aβ toxicity triggered progression of body paralysis in the *CL4176* worms. AD worms treated with CBE in various ways resulted in reduced body paralysis as compared to control worms at each time point. CBE preventive treatment showed significantly greater efficacy than it of therapeutic treatment, but similar to long-term treatment (n.s.). Each paralysis assay was conducted in triplicates and repeated at least three times with similar results. “% Non-paralyzed” indicates the average non-paralysis among the multi-replicates and error bars represent the standard deviation. *P* value was calculated using Student’s *t*-test. *P* < 0.05 vs controls. **P** < 0.01 vs corresponding controls; n.s. represents no significance

**CBE preventive treatment results in more reduction of Aβ expression in AD worms than therapeutic treatment**

The expression level of Aβ peptides determines the Aβ toxicity that triggers body paralysis in AD worms. Given that the preventive treatment with CBE showed greater efficacy in delaying the progression of paralysis than therapeutic treatment, we determined whether the stronger anti-AD efficacy was ascribed to the greater reduction of Aβ expression in AD worm. We performed quantitative real-time PCR (qRT-PCR) to measure and compare Aβ expression in AD worms treated with CBE in prevention and therapy protocols (Fig. 3A). The *CL4176* worms without CBE treatment served as negative control and AD worms always exposed to CBE starting from eggs (long-term treatment) served as positive controls. Impressively, our positive control worms showed 69.6% reduction of Aβ expression, compared with the negative controls. In contrast, preventive treatment and therapeutic treatment resulted in 53.5% and 37.4% reduction of Aβ expression, respectively, relative to negative controls. As the preventive treatment occurred before Aβ was expressed, the 53.5% reduction of Aβ expression was fully ascribed to the preventive efficacy of CBE. Compared to the 69.6% reduction of Aβ expression in positive control worms, our data further supported the idea that CBE-mediated anti-AD effect may largely depend on its preventive effect.

After examining Aβ expression at the transcriptional level by qRT-PCR, we also performed Western blot analyses to measure Aβ expression at protein level in AD worms treated with CBE (Fig. 3B). Our results indicated that the total amount of Aβ species in AD worms with long-term treatment was around 37.4% less than that in the control AD worms, while preventive treatment and therapeutic treatment resulted in 25.3% and 17.5% reductions, respectively.

**CBE treatment affects AD worms’ memory**

Considering that the memory degeneration is generally
Fig. 3  CBE treatments significantly repress Aβ expression in AD worms. (A) The RNA level of Aβ was quantified using qRT-PCR in transgenic CL4176 worms treated with CBE in different ways (normalized to act-1). Results are the average of three independent experiments, and error bars are standard error of the mean. * P < 0.05 vs the non-treated control; ** P < 0.01 vs the non-treated control. (B) Western blot analysis was conducted to measure the level of Aβ proteins. Image-J software was engaged to quantify Aβ proteins. The graph shows the mean intensity of Aβ proteins and is the result of three independent experiments. P value was calculated using Student's t-test. * P < 0.05 vs controls

associated with AD, CBE's anti-AD properties urged us to examine whether CBE supplementation may protect AD worms' memory from degeneration when Aβ was expressed. The C. elegans habituation to bacterial foods was utilized as an indicator to evaluate the health status of AD worms' memory [27-31]. Briefly, CL4176 worms maintained on OP50 and seeded NGM plates were synchronized by laying eggs on either regular NGM plates or CBE containing NGM plates at 16 °C. When eggs were hatched and developed into L3 stage, the worms were washed and transferred to the worm area on corresponding assay plates and upshifted to 25 °C to induce Aβ expression. The assay plates setup is described in Fig. 4A. Our results showed that none of AD worms without exposed to CBE (negative control) migrated into OP50 food area, while 19.4% worms treated with CBE all the time from egg until paralysis (long-term treatment, positive control) migrated into OP50 food area (Fig. 4B, Table 1). These findings suggested that CBE supplementation may be potent to protect AD worms from memory degeneration.

In contrast, relative to negative controls, CBE preventive treatment and therapeutic treatment resulted in 13.4% and 6.6% AD worms migrating into OP50 food area, respectively (Fig. 4B, Table 1). Given that CBE preventive treatment displayed more potency to delay AD worms' body paralysis relative to therapeutic treatment, fewer worms in food area for therapeutic treatment may be due to the motility defects rather than memory protection. To rule out this possibility, we further analyzed the number of worms in the worm area. Theoretically, if the preventive treatment and therapeutic treatment did not have a significant difference to influence worms’ memory, a similar number of worms should move out of the worm area and migrate towards the food area at the early stage of Aβ expression. However, our results indicated that only 11.9% AD worms with therapeutic treatment moved out of the worm area, while 22.4% AD worms with preventive treatment moved out of the worm area towards the food area (Fig. 4C, Table 1). These findings clearly demonstrated that CBE preventive treatment contributed more to CBE-mediated memory protection as compared to therapeutic treatment.

Taken together, our findings from the memory assay also implied that CBE supplementation, if applied as an AD preventive agent, may significantly improve the healthy aging in aged populations. Undoubtedly, to support this hypothesis, further experiments in mammals should be performed.

In summary, the preventive treatment with CBE significantly delayed the body paralysis of C. elegans AD model by alleviating Aβ toxicity. On basis of our results, we were the first to suggest how to implement CBE to optimize its anti-AD effects in a multicellular organism, i.e., to delay the onset of AD or ease the symptom of AD, CBE supplementation should start early before Aβ toxicity is accumulated and it is better if the
Fig. 4 Influence of the long-term treatment, preventive treatment and therapeutic treatment on *C. elegans* memory in CL4176. (A) A scheme of the setup of memory assay plates. (B) CBE treatment of prevention, cure and long term led to more worms moving to food area when compared to control worms without CBE treatment, indicating the improved memory of AD worms. (C) Relative to therapeutic treatment, AD worms after the preventive treatment showed an elevated desire to move out of worm area toward food area, suggesting the more potency of preventive treatment on memory health. Each memory assay was conducted in triplicates and repeated at least three times with similar results. "% worms" indicates the average percentage of worms among the multi-replicates and error bars represent the standard deviation. *p* value was calculated using Student’s *t*-test. *P* < 0.05, **P** < 0.01 vs the control.

<table>
<thead>
<tr>
<th>CBE treatment</th>
<th>OP50 food area</th>
<th>Blank area</th>
<th>Worm area</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No CBE</td>
<td>0</td>
<td>3</td>
<td>167</td>
<td>170</td>
</tr>
<tr>
<td>Preventive</td>
<td>18</td>
<td>12</td>
<td>104</td>
<td>134</td>
</tr>
<tr>
<td>Therapeutic</td>
<td>10</td>
<td>8</td>
<td>133</td>
<td>151</td>
</tr>
<tr>
<td>Long-term</td>
<td>26</td>
<td>30</td>
<td>78</td>
<td>134</td>
</tr>
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</table>

This data represents the average of three independent trials for each test.

CBE treatment is applied in a long-term manner. Considering that CBE-mediated beneficial effects on healthspan require evolutionarily conserved mechanisms among diverse species ranging from *C. elegans* to mammals [18, 32-33], it is very likely that CBE’s anti-AD effects may also act through those highly conserved signaling pathways. Thus, our findings set up a foundation to study if the similar results would occur in higher order organisms, with an imperative implication for the application of CBE in AD treatment.

**Conclusion**

By comparing CBE’s efficacy of prevention and therapy in AD treatment in the present study, we found that CBE applied as a preventive agent, rather than a therapeutic medicine, was more effective. Certainly, it is of interest to further investigate whether the similar results would occur in humans. The preventive treatment with CBE significantly delayed the body paralysis of *C. elegans* by alleviating Aβ toxicity. To our best knowledge, this was the first to suggest that, to delay the onset of AD or ease the symptom of AD, CBE supplementation should start early before the Aβ toxicity was accumulated and the CBE treatment is better applied in a long-term manner. Considering that CBE-mediated beneficial effects on healthspan require evolutionarily conserved mechanisms among diverse species ranging from *C. elegans* to mammals [18, 32-33], it is very likely that CBE’s anti-AD effects may also act through those highly conserved signaling pathways. In conclusion, our findings paved a foundation for future study to determine if the similar results would occur in higher order organisms, which may have an imperative implication in AD treatment.

**Acknowledgements**

The authors are grateful to other members of the Dong Laboratory for helpful discussions. We thank Naturex-DBS, LLC (Sagamore, MA, USA) for providing cranberry extract.

**References**


Cite this article as: GUO Hong, DONG Yu-Qing, YE Bo-Ping. Isoquercitrin suppresses the expression of histamine and pro-inflammatory cytokines by inhibiting the activation of MAP Kinases and NF-κB in human KU812 cells [J]. Chin J Nat Med, 2016, 14(6): 427-433.